A NEW TYPE OF INACTIVATION OF STREPTOMYCIN BY *E. COLI*

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(Received for publication July 3, 1976)

Previously described cases of streptomycin inactivation by R-factor carrying strains of *E. coli* have not lead to any measurable decrease in antimicrobial potency in the bulk substrate toward the culture. In these cases each cell inactivates only a few molecules. Out of 1,800 strains of *E. coli* we have isolated five strains which inactivate streptomycin in large amounts giving a final concentration of the inactivation product of 0.25 mg/ml in 36 hours. Unlike all other streptomycin-resistant strains investigated these five strains were sensitive to butyl-streptomycylamine, a streptomycin derivative acting in the same way as streptomycin. The crude inactivation product has been isolated. Inorganic phosphate is liberated by treatment with alkaline phosphatase resulting in a streptomycin-like compound without any antimicrobial activity.

Streptomycin resistance in clinically important bacteria is most frequently caused by enzymatic inactivation of streptomycin (SM) and dihydrostreptomycin (DHS). The active enzymes are bound to the membrane of the bacteria. However, when SM (DHS) is added to a culture of such a bacterium, no decrease in antimicrobial activity in the broth can be measured. This can be explained by assuming that only a few molecules of SM (DHS) enter the R-factor carrying cell where they are inactivated by the membrane enzyme systems. The inactivating process causes a change in the structure of the membrane which becomes impermeable to SM (J. DAVIES, personal communication 1974). Strong evidence in favour of this hypothesis has recently been published by LUNDBÄCK and NORDSTRÖM who found a low uptake of SM (DHS) by R-factor carrying SM-resistant *Escherichia coli* compared to normal sensitive strains.[1]

SM contains a hydrated aldehyde group and many derivatives have been made by reactions with this group. The derivatives thus obtained vary in activity from that of SM to complete inactivity. Streptomycylamines (SM-amines) exhibit varying potencies depending on the test organism and the amine side chain[2]. These compounds have been studied recently by HEDING and DIEDRICHSEN[3] who found that the higher homologues of the SM-amines (primary amines) are active towards SM-sensitive and ribosomal resistant strains of *E. coli* and that the mode of action of these SM-amines is different from that of SM.

The short chain SM-amines are less active towards our sensitive strain no. 079 and inactive towards the mutant strains (ribosomal). The mode of action of the short-chain SM-amines appeared to be the same as that of SM.

An investigation of the activities of butyl-SM-amine (short chain) and dodecyl-SM-amine (long chain) towards 30 randomly chosen highly resistant clinically isolated *E. coli* strains (>400 μg/ml) is presented in this paper.

Materials and Methods

Bacterial strains: The investigated strains of *E. coli* were selected among SM-resistant strains isolated through daily routine investigation of the material sent in for bacteriological investigation and sensitivity testing at Statens Seruminstitut.

The strains were at first selected through a disc diffusion method using nutrient agar with 5 % horse blood and paper discs (diam. 6 mm) with a content corresponding to 40 μg SM and 20-hour pre-diffusion. The prediffused plates were inoculated and incubated overnight at 36°C. Resistant strains showed inhibition zones less than 6 mm in diameter. The isolated resistant strains were investigated for growth ability in nutrient broth with a content corresponding to 400 μg SM-base/ml. Only those strains fulfilling this criterium were taken into further investigations.

Antibiotics: DHS from NOVO A/S Copenhagen. Butyl-SM-amine and dodecyl-SM-amine were prepared as described earlier[3].

Quantitative measurement of antimicrobial activity (Minimal inhibitory concentration (MIC)) of SM-derivatives: The MIC-values of DHS, butyl-SM-amine and dodecyl-SM-amine were estimated by the conventional two-fold dilution method in nutrient assay broth (3 g beef-extract and 5 g trypton per liter) at pH 6.8. The values were read after 24 and 48 hours and the MIC-value is the lowest concentration giving no growth. The variation between the first and last reading was never more than one dilution.

Bio-assay: The concentration of DHS in the culture medium was determined by an agar-diffusion method using *Bacillus subtilis* ATCC 6633 as test-organism[4]. Before testing, one drop of HCl (2 N) was added to the sample and the cells were removed by centrifugation.

Thin-layer chromatography (TLC) and high voltage electrophoresis was performed as described by H. HEDING[5] and H. HEDING & K. BAJPAI[6].

Isolation of the inactivation product: A fermentation was performed in a 10-liter fermentor. The cells were removed by centrifugation giving a solution containing the inactivation product and some unmodified DHS. This solution was treated with a weak cation-exchanger (IRC 50, Hopkin and Williams Ltd., England) at pH 7.0 to which DHS is bound and thereby removed. The inactivation product was isolated from the resulting solution by a strong cation-exchanger (IR 120, Hopkin & Williams Ltd., England) at pH 3.0. The inactivation product was liberated with 25 % ammonia water at pH 11.0. This solution was evaporated and the residue was dissolved in methanol. The product was isolated by precipitation with 8 volumes of acetone.

Hydrolysis of the inactivated DHS was carried out with alkaline phosphatase (Sigma, St. Louis, U.S.A.) at pH 10.4 in a 0.01 M tris-buffer (0.01 tris, 0.014 M $Mg(OAc)_2$, 0.06 M KCl and 0.006 M mercaptoethanol). To avoid bacterial contamination 0.1 % toluene was added. The reaction was carried out at 37°C and followed by electrophoresis of the reaction mixture. Chemical hydrolysis was performed by treatment with 0.01 N HCl at 95°C. Five minutes treatment lead to approximately 50 % hydrolysis without liberation of detectable amounts of streptidine.

Results and discussion

Out of 1,800 *E. coli* strains investigated, all from urinary tract infections, only 9 % (160) were SM-resistant. The sensitivity pattern for 30 SM-resistant strains chosen at random is shown in Table 1.

The table shows that all the strains are unaffected by DHS concentrations less than 250 μg/ml at pH 6.8. All the strains except one were susceptible to 16 μg/ml of dodecyl-SM-amine.

Table 1. Sensitivity pattern for the SM-resistant strains of *E. coli*. Estimated at pH 6.8.

Strain *E. coli*	MIC (μg/ml)		
	DHS	Butyl-SM-amine	Dodecyl-SM-amine
U 3012/74	500	250	8
U 3060/74	250	125	31
U 3125/74	250	125	8
U 3137/74	250	500	16
U 3226/74	250	125	16
U 3229/74	>1,000	63	8
U 3279/74	500	16	8
U 3285/74	500	16	16
U 3352/74	>1,000	250	8
U 3407/74	1,000	16	8
U 3512/74	>1,000	250	8
U 3536/74	>1,000	63	16
U 3554/74	500	500	4
U 3608/74	500	500	16
U 3610/74	500	250	8
U 3640/74	500	500	8
U 6488/74	500	250	8
U 6721/74	>1,000	16	8
U 6856/74	>1,000	16	16
U 7489/74	>1,000	63	8
U 7503/74	>1,000	250	16
T 3373/74	500	63	8
T 3428/74	500	500	16
T 3737/74	>1,000	125	16
T 3790/74	1,000	125	8
T 3843/74	1,000	63	8
T 3889/74	1,000	63	16
T 8041/74	1,000	63	8
T 8528/74	1,000	125	8
T 8652/74	1,000	125	8

Only five strains were sensitive to butyl-SM-amine (MIC=16 μg/ml) while the others were resistant.

TLC and electrophoresis of the broth of the first glass in the serial dilution test with visible growth was performed. Except for the five strains sensitive to butyl-SM-amine no modified DHS could be detected. However, these five strains had all converted DHS into a biological inactive compound ($\sim$50 % conversion) with approximately one third of the mobility of DHS in the electrophoresis system but with the same Rf-value in the TLC system. This is to the best of our knowledge the first example of substantial inactivation of SM (DHS) by *E. coli* described.

Two inactivation products have been described, 3-phosphate[7] and 3″-adenylate.[8] According to the literature these substances are, however, not excreted into the medium, and no detectable decrease in the concentration of SM (DHS) in this can be measured as mentioned above.

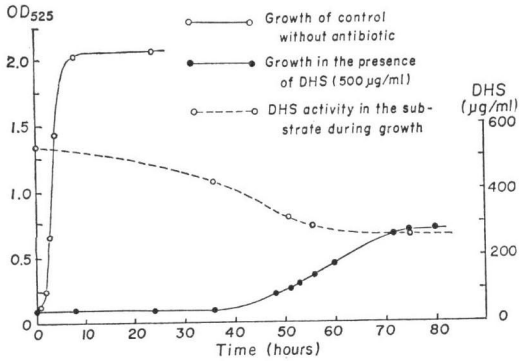
Our strain no. U 3279/74 inactivates DHS totally when the concentration is low (10$\sim$50 μg/ml). Addition of larger amounts of antibiotic (500 μg/ml) leads to about 50 % inactivation.

Fig. 1 shows a growth experiment with strain no. U 3279/74. On a DHS -free nutrient broth substrate a typical growth curve for *E. coli* appears. The exponential phase lasts only for a few hours and maximal cell mass (OD_{525}=2.15) is reached in approximately 10 hours. When DHS is added (500 μg/ml) at the time of inoculation no visible growth occurs for 36 hours. During this lag period DHS is inactivated. The inactivation is not complete and it stops at the middle of the exponential growth phase (55 hours). The maximal cell mass obtained is less than in the control experiment. The number of cells/ml may however be the same in the two cases (not estimated).

In another experiment DHS was added to a culture of the same strain during the first part of the exponential growth phase. The growth continued undisturbed and no inactivation product could be detected (not shown).

The chemical structure of the inactivation product is not yet fully elucidated but the following characteristics have reproducibly been found. The compound is hydrolyzed by alkaline phosphatase under liberation of inorganic phosphate and a product which in our

Fig. 1. The time dependence of growth and inactivation.
Organism: *E. coli* U 3279/74.



TLC and electrophoresis systems can not be distinguished from DHS—it is however devoid of antibacterial activity (*E. coli*, *B. subtilis*).

Mild acid hydrolysis gives the same result. Liberation of phosphate and a product which except for the lack of antimicrobial activity appears to be DHS.

Conclusion

The pattern of resistance for a series of clinical isolated SM-resistant *E. coli* has shown that only five out of these were sensitive to butyl-SM-amine, and only these five strains could inactivate DHS in large amounts. The correlation between these two features is highly significant.

The five strains all inactivated SM (DHS) in large amounts (0.25 g per liter). The sensitivity to butyl-SM-amine can be explained by steric hindrance of the inactivating enzyme system. As all other SM-resistant *E. coli* also are resistant to the butyl-SM-amine derivate we conclude that the enzyme system of these strains is different from the SM-phosphotransferase and SM-adenyltransferase previously described. This hypothesis is further supported by the fact that the inactivated DHS we have isolated does liberate phosphate by treatment with alkaline phosphatase or acid but without restoration of activity. The previously described inactivation products are according to the literature[9] reactivated by such treatment.

The inactivation experiments can be explained by assuming a defect in the inactivation model proposed by JULIAN DAVIES (personal communication 1974). The enzyme is membrane-bound and SM (DHS) is inactivated at this site under a simultaneous change of the membrane structure whereby further entrance of SM(DHS) is excluded. If the last step in this sequence is defective in our inactivating strains, the inactivation would continue and the product would accumulate in the medium as reported here.

The extremely long lag phase (36 hours) is explained by the same assumption. To explain why the inactivation stops during the first part of the exponential growth phase further experiments are needed.

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